Plant Cell, Tissue and Organ Culture (PCTOC): Journal of Plant Biotechnology

Co-expression of onion chalcone isomerase in Del/Ros1-expressing tomato enhances anthocyanin and flavonol production

--Manuscript Draft--

Manuscript Number:	PCTO-D-16-00226R1
Full Title:	Co-expression of onion chalcone isomerase in Del/Ros1-expressing tomato enhances anthocyanin and flavonol production
Article Type:	Original Article
Keywords:	anthocyanins; flavonol; chalcone isomerase; tomato; Delila; Rosea1; Solanum lycopersicum; secondary metabolite
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Funding Information:	HIGreen System (000) — Dr Wansang Lim
Abstract:	Anthocyanins are colorful pigments known for contributing antioxidant effects to the human diet which provide health benefits that protect against several forms of cancer and vascular disease. Unfortunately, tomatoes found in nature have very low anthocyanin content. Anthocyanin rich, purple tomatoes by the ectopic co-expression of two transcription factors Delila (Del) and Rosea1 (Ros1) from the snapdragon Antirrhinum majus have been generated. However, the Del/Ros1 (DR)-expressing tomatoes cannot sufficiently upregulate all necessary key endogenous genes, particularly chalcone isomerase (CHI), for full utilization of the anthocyanin production pathway. In this study, CHI from onion Allium cepa was introduced into DR-expressing tomatoes for a further increase of anthocyanin levels in both the peel and the flesh of tomatoes. We achieved up to 400-fold and 260-fold increases in the levels of anthocyanins in tomato peel and flesh, respectively, in CHI/DR transgenics compared with 100-fold and 80-fold increases in tomato peel and flesh in the DR only expressing lines. Furthermore, CHI/DR-expressing tomatoes increased up to 200-fold more total flavonol content in flesh compared with the wild-type tomatoes. In summary, stacking CHI with DR could significantly increase the levels of anthocyanins and flavonols in tomato fruit.
Response to Reviewers:	Dear Editor, Thank you and the reviewers' great efforts and time to review our manuscript! Thanks a lot for the constructive suggestions! We have taken a lot of time to do the thorough revision for the manuscript. The following parts are the point-by-point responses. Thank you very much!

Wansang

Editor-in-Chief:
In agreement with comments and queries by the reviewers and the advice from the Associate Editor, before this article may become acceptable the authors should undertake a major and thorough revision taking into account all concerns raised and they should accompany the resubmission with a detailed written point-by-point response.
AU: We have taken a lot of time to do major revision for this manuscript and provided point-by-point responses as below.

Associate Editor
This paper is interesting, but some work is still necessary in order to become acceptable. Both reviewers recommend an extensive list of changes. Most of them are related to formal aspects, and therefore are easy to do. Others, however, relate to more conceptual aspects, and authors should pay special attention to fix them. Some of these major aspects to be addressed include the presentation of results in the text, a sharp distinction between their own results and results from previous literature, and the general organization of the manuscript. When ellaborating the manuscript, little attention has been paid to the "instructions for authors" of this journal. Just some examples: formatting of headings and subheadings has nothing to do with what is supposed to be done for a PCTOC manuscript, there is no list of figure legends, the figures are all included in Microsoft Word independent file, there is no key message, instead of the key message they included the cover letter, and
finally, the cover letter seems written to the editors of Functional Plant Biology! In summary, this manuscript must be profoundly remodelled before acceptance.

AU: according to the comments from you and the reviewers, we did a lot of modifications from aspects of formats and content. For example, we carefully read the "instructions for authors", and made required changes for the formats.
- Change heading and subheading - Corrected
- Figure legend – Added
- Independent file – Independent file added besides word file.
- key message – It is added.

PCTO-D-16-00226

The paper describes the effects of co-expression of three pathway genes on anthocyanin and flavonol production. The work is interesting but more care needs to be taken in presentation of the results and distinguishing between what the authors have found and what was previously known from the literature. The following specific comments must also be addressed.

Introduction: Some sentences are too long. Please re-read and shorten sentences which are difficult to follow.
AU: we did major revision for the introduction to make the sentences short and clear.

Line 38: The authors mention ‘another bioactive flavonoid’ when no specific mention of flavonoids has yet been made. Therefore, please first tell the reader that anthocyanins are flavonoids
AU: corrected.
- In line 33, dietary phenolics is changed to dietary flavonoids.

Line 42-47: Sentence repeats itself
AU: re-wrote to avoid repeat.

Line 42 & 48: Repeat each other.
AU: removed repeated sentences in the line 48 and line 49.

Line 60: What do the authors mean by ‘without the expense of lycopene’? Please clarify.
AU: corrected.
– Flavonoids and terpenoid which the lycopen belong to has common precursor (Kang, McRoberts et al. 2014). They can compete with each other. However, we achieved increase of flavonoids without decrease of lycopen content. We removed the sentence.

Line 70: ‘bundler’ should be ‘bundle.’
AU: corrected.
Line 81: ‘which’ should be ‘and.’
AU: corrected.

Line 86:“transgene” should be “transgenic”
AU: corrected.

Line 89 to end: Sentence is confusing. What do the authors mean by ‘this biotechnology?
AU: re-wrote these several sentences to make it clear.

Materials & Methods: Use of past tense is not consistent.
AU: corrected.

Line 112: What selectable agent was used for selection during plant regeneration?
AU: The selectable agent information is added.

How many replications (# plants, # fruits) and what were the sample sizes for the antocyanin, lycopene, antioxidant and other measurements? What was the zygosity of the analyzed plants?
AU: I add “For repetition, each line had 4-6 plants. The two to three pooled tomatoes were collected from each plant for every lines.” in statistical analysis section.

- We used the cultivar Rubion. And we maintained the line by selfing for at least five years by every three months. We believe it is homozygous. I added the cultivar name in “Plant transformation” section.

Line 133: “The extraction procedure was following (Muir et al. 2001)”should be corrected.
AU: corrected.

Line 142: Sentence is not clear.
AU: The chemicals in eluant B are H2O, C4H10O and CH3COO. And the ratio of them is 330ml, 8ml and 1ml.

“Total flavonoid and anthocyanin content” section should have a reference or should contain an explanation for the nanodrop measurement of total flavonoid content.
AU: We added the reference
.
Line 164: why is lettuce mentioned?
AU: We corrected

Results:
The zygosity of the lines that were examined for fruit weight and flavonol traits is unclear. Please report the zygosity for each inserted gene. Were hemizygous and homozygous for transgene lines treated the same? The authors must clarify if they did or did not select for homozygosity of the transgenes when using F2 individuals derived from the T1 crosses.

AU: Consistent with a single insertion, the T1seeds of these lines showed a segregation pattern of 3 : 1. To obtain homozygous, segregation analysis on T2 seeds from self-pollinated T1 plants was carried out, and the expression was examined by reverse transcriptase (RT)-PCR analysis. The seeds from homozygous individual plants were used for crossing. We found that there was no difference of fruit weight, flavonol and flavoonids content between homozygous and hemizygous plants for every

gene.

Also, the actual concentrations of each compound should be reported in addition to the fold changes that were observed. This applies to the entire Results section.
AU: – The concentration is added.

Line 224: What do the authors mean by 'liking' of color?
AU: this part was deleted, as this result was coming from my published paper.

Line 298: Average fruit weight and number are given but it is not clear for which line.
AU: - It is average of all across the lines because there is no statistical difference between lines. I cleared it.

Table 1: Standard error is indicated in column but footnote refers to SD. Table should indicate results of statistical analysis.
AU: - It is standard deviation. I corrected.

Discussion
In discussion section roles of the genes in flavonol and anthocyanide biosynthetic pathways were discussed. But for some of this information no proper references were provided. For example in line 339 "DR upregulates F3'5'H, 340 which converts dihydrokaempferol to dihydromyricetin.". Because of this problem the reader cannot follow which claims you inferred from your experimental data or were known from previously conducted studies in the literature.
AU:- I inserted citation

Line 304: 'to the' should be 'in the'.
AU: corrected.

Line 309: It is claimed DR has less impact on flavone accumulation than CHI. This claim should be backed up with experimental (numerical) data.
AU: We added numerical data: "The CHI alone lines increased the flavonol and anthocyanin content in peel by average 8 and 1.4 fold, respectively, however the DR alone lines increased them by only 4 and 77 fold (Fig 5), respectively".

Line 320. CHS is stated to have the biggest role in flavonol production. What is the basis of that claim, previous studies or your own data?
AU: We added a reference.

Line 322: The sentence starting that line claiming that DR over-expressing lines has purple fruits without any upregulation of FLS or CHS. What is the basis of that claim, previous studies or your own data? Claims mentioned in the following two sentences should be clarifed as well.
AU: We clarified the statement to "…the flesh of our DR overexpressing tomatoes was visibly purple in this experiment without upregulation of CHS and FLS (Butelli et al. 2008)…"

Line 329: Reference needed.
AU: The reference is added.

Line 330: "These genes and our DR/CHI do not compete, instead, they work together." How can you conclude that the DR CHI, PAL, and F3H work together without providing any gene expression data in your study or giving any reference from previous studies.
AU: this claim was deleted, as this is speculation and we don't have references.

line 333: Sentence cannot start with parentheses.
AU: corrected.

Line 339: "DR upregulates F3'5'H, which converts dihydrokaempferol to dihydromyricetin." Requires a reference.
AU: The reference is added.

Line 348: "delphididine" should be "delphinidine"
AU: corrected.

	Line 359: “solution” should be “extract” AU: corrected.

Co-expression of onion *chalcone isomerase* in *Del/Ros1*-expressing tomato enhances anthocyanin and flavonol production

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Abstract

Anthocyanins are colorful pigments known for contributing antioxidant effects to the human diet which provide health benefits that protect against several forms of cancer and vascular disease. Unfortunately, tomatoes found in nature have very low anthocyanin content. Anthocyanin rich, purple tomatoes by the ectopic co-expression of two transcription factors *Delila* (*Del*) and *Rosea1* (*Ros1*) from the snapdragon *Antirrhinum majus* have been generated. However, the *Del/Ros1* (*DR*)-expressing tomatoes cannot sufficiently upregulate all necessary key endogenous genes, particularly chalcone isomerase (*CHI*), for full utilization of the anthocyanin production pathway. In this study, *CHI* from onion *Allium cepa* was introduced into *DR*-expressing tomatoes for a further increase of anthocyanin levels in both the peel and the flesh of tomatoes. We achieved up to 400-fold and 260-fold increases in the levels of anthocyanins in tomato peel and flesh, respectively, in *CHI/DR* transgenics compared with 100-fold and 80-fold increases in tomato peel and flesh in the *DR* only expressing lines. Furthermore, *CHI/DR*-expressing tomatoes increased up to 200-fold more total flavonol content in flesh compared with the wild-type tomatoes. In summary, stacking *CHI* with *DR* could significantly increase the levels of anthocyanins and flavonols in tomato fruit.

Keywords

Tomato · Chalcone isomerase (CHI) · Del/Ros1 · anthocyanin ·flavonol

Introduction

Anthocyanins are a subgroup of dietary flavonoids that are responsible for the purple color of various fruits and vegetables. They have been associated with potentially health-beneficial effects in various diseases, such as cardiovascular disease (Lee et al. 2007; Wallace 2011), diabetes (Ghosh and Konishi 2007), and cancer (Seeram et al. 2006; Shih et al. 2005). In addition, anthocyanins have the capacity to modulate cognitive and motor functions, enhance memory, hinder obesity, and prevent age-related decline (Cho et al. 2003). Another bioactive flavonoid, rutin is the most abundant flavonol in tomatoes (Colliver et al. 2002), albeit present in very small amount. Rutin also exhibits a range of pharmacological effects such as anti-oxidation, anti-inflammation, and anti-hypertension as well as vasoconstrictive, spasmolytic, and positive inotropic effects (Kuntic et al. 2011; La Case et al. 2000; Landberg et al. 2011). Although tomato is one of the most popular fruits used in many diets, it has a very low overall anthocyanin and flavonol content. Therefore, to improve health-beneficial effects of tomato, increasing the total anthocyanin and/or flavonol content of tomatoes has been an important objective (Butelli et al. 2008; Muir et al. 2001).

Flavonols, including rutin, are present mostly in peel of the tomato, while only traceable amounts are present in the flesh. Similarly, tomato peel has a very small amount of anthocyanin and the flesh has almost no anthocyanin. Flavonoid pathway not active in the fruit flesh is due to the expression of specific flavonoid biosynthetic genes are down-regulated in this tissue (Bovy et al. 2002; Colliver et al. 2002; Verhoeyen et al. 2002). This is a stark contrast to other fruits of the *Solanaceae* family such as eggplant and pepper (Zuluaga et al. 2008).

The red color that tomatoes and some peppers exhibit at the ripening stage is due to the carotenoid lycopene rather than anthocyanin. Most health beneficial effect of tomato is due to lycopene. Because tomato contains lycopene, it is a great strategy to increase anthocyanin content so that they can provide health benefit to consumers with both lycopene and flavonoid.

Heterologous overexpression of either the structural or regulatory genes in the flavonoid pathway has been used to increase flavonoid levels or modify the flavonoid profile in tomatoes (Gonzali et al. 2009). For example, the concurrent overexpression of both regulatory snapdragon genes *Delila* (*Del*) and *Rosea1* (*Ros1*) in tomatoes results in a marked increase in anthocyanin content (Butelli et al. 2008). This can be observed visually as the fruit develops and ripens into a purple color. Moreover, an overall increase in flavonol levels in tomato fruits has been achieved

by simultaneous overexpression of the maize transcription factors *Lc* and *C1* (Bovy et al. 2002). The Arabidopsis *MYB75*/*PAP1* transcription factor is also effective in specific local cells in the epidermal or cortical region or in proximity to a vascular bundle but is not quite as effective as in the fruit (Zuluaga et al. 2008). *AtMYB12* led to increased polyphenol content by as much as 10% in the dry weight of tomato fruits (Luo et al. 2008). Each of these regulatory genes has a unique effect on the flavonoid profile of the fruit they are introduced into.

As an alternative approach, the concurrent overexpression of structural genes *CHI* (chalcone isomerase), *CHS* (chalcone synthase), *F3H* (flavanone-3-hyudroxylase) and *FLS* (flavonol synthase, Fig 1) increases levels of flavonols in tomato flesh, demonstrating that structural genes also play important roles in regulation of flavonoid synthesis pathway (Verhoeyen et al. 2002). In addition, an increase of up to 78-fold flavonol content in transgenic tomato peel was observed by overexpression of the petunia *CHI* gene. The results indicated that the conversion of narengenein chalcone to narengenin is a rate-limiting step in flavonol biosynthesis in the peel and the overexpression of *CHI* alleviates a major bottleneck and causes a significant increase in the levels of the flavonols (Muir et al. 2001).

Heterologous overexpression of *Del* and *Ros1* (*DR*) in tomatoes increases the activity of endogenous *CHI*, but not sufficiently to resolve the bottleneck (Butelli et al. 2008). Since CHI is a major rate-limiting step in flavonol biosynthesis, we hypothesize that co-expressing *CHI* in the *DR*-expressing transgenic tomato lines could fully maximize anthocyanin and flavonol production pathway. In this study, *CHI* from onion (*Allium cepa* L.) was isolated and transformed to generate *CHI/DR*-co-expressing tomato plants. The stacked lines exhibited significant increases in both flavonol and anthocyanin content in both the peel and the flesh. The sustained growth of *CHI/DR*-co-expressing tomatoes demonstrates that this strategy could improve tomato nutritional content significantly to make tomato a healthier diet.

Material and Methods

Vector construction

CHI gene was cloned from red onion (accession number, AY700851.1) (Kim et al. 2004). RNA was extracted using an RNeasy plant mini-kit from QIAGEN (Valencia, CA, U.S.A). cDNA was made with the Advantage RT-for-PCR Kit from Clontech (Mountain View, CA, U.S.A). The primer sequences for *CHI* were forward 5' -ATGGAAGCAGTGACAAAGTT -3', reverse 5'-T CATGAAAGCACCGGTAACT-3'. The PCR product was inserted to pE1775 expression vector (Lee et al. 2007) . The pE1775 vector harboring *CHI* gene was transferred to *Agrobacterium* (LBA4404) using the freeze-thaw method (Holsters et al. 1978). The Del/Ros1 vector harboring *Del/Ros1* gene was used for this study (Butelli et al. 2008).

Plant transformation

Seeds of the *Solanum lycopersicum* L. were surface-sterilized and germinated on a Murashige and Skoog inorganic salt medium (Murashige and Skoog 1962). Tomato transformation was performed via the *Agrobacterium*-mediated transformation method using cotyledon and hypocotyl explants (Park et al. 2003). The selectable agents for *CHI* and *DR* harboring vectors were hygromycin (Lee et al. 2007) and kanamycin(Butelli et al. 2008), respectively. *Agrobacterium tumefaciens* LBA4404 was used in generating stable transgenic plants. The plasmids containing *CHI* and *Del/Ros* were introduced into *A. tumefaciens* using the freeze-thaw method (Holsters et al. 1978). Following inoculation with *A. tumefaciens*, the plant cultures were maintained at 25 ˚C under a 16-h photoperiod. After 6 to 8 weeks, regenerated shoots were transferred to a rooting medium for 6 additional weeks. The greenhouse temperature was maintained within a range of 25 ˚C to 30 ˚C (Lim et al. 2014). The cultivar used for all transformation was Rubion maintained by selfing.

Molecular analysis of transgenic plants

Tomato genomic DNA and RNA were extracted from leaf tissue with the Qiagen Plant DNA extraction kit. Tomato RNA was extracted from the peel and flesh with the Qiagen Plant RNA extraction kit. cDNA was synthesized using moloney murine leukaemia virus-reverse transciptase (BD Biosciences Clontech, Palo Alto, CA, USA). All polymerase chain reactions

(PCR) were performed with a GoTaq Flexi DNA Polymerase kit (Promega Corporation, Madison, WI, USA).

HPLC analysis

One gram of peel was frozen in liquid nitrogen and macerated in a 15 ml round-bottom tube with a plastic pestle. The samples were hydrolyzed with 4.8 ml of 62.5% methanol and 1.2 ml 6M HCl for 60 min at 45˚C. The extracts were cooled on ice and sonicated at temperature for 45 min and then centrifuged at 13,000 RPM for 20 min. Supernatant filtering was performed with a 0.45 μm filter. The extraction procedure was following a published protocol (Muir et al. 2001).

HPLC analysis was modified from a published paper (Lim et al. 2014). The HPLC system includes an autosampler (SpectraSYSTEM AS1000, Thermo Separation Products, San Jose, CA, USA), a pump (HP 1050, Hewlett Packard, Palo Alto, CA, USA), an integrator (HP 3396, Hewlett Packard, Palo Alto, CA, USA), and a UV/VIS detector (Acutect 500, Thermo Separation Products, San Jose, CA, USA). A 5 μL sample was injected into the HPLC column (Discovery BIO Wde Bore C18, 15 cm x 4.6 mm, 5 μm, Supelco, Inc., Bellefonte, PA, USA) with a guard column (Discovery BIO Wide Bore C18, 2cm x 4mm, 5 μm, Supelco, Inc., Bellefonte, PA, USA). The sample was eluted with eluant A [H_2O/ CH_3COOH (338/1, v/v)] and eluant B [H_2O/$C_4H_{10}O$/CH_3COOH (330/8/1, v/v/v)] at a flow rate of 1.8 mL/min. The gradient is A 20 ~ 20%, B 80 ~ 80%, 0~5 min: A 20 ~ 0%, B 80 ~ 100%, 5 ~ 25 min. Qualitative identification of flavonoid peaks was determined by co-chromatography (equivalent retention time) with chemically pure standards (5 mg/100 mL), and quantification was based on the integration of the peak area compared with a standard curve. The standards are quercetin-3-O-glucoside (Sigma–Aldrich, St. Louis, MO, USA), rutin (Sigma–Aldrich, St. Louis, MO, USA), Kaempferol rutinoside (Sigma–Aldrich, St. Louis, MO, USA), Quercetin (Sigma–Aldrich, St. Louis, MO, USA), Naringenin (Sigma–Aldrich, St. Louis, MO, USA), and Naringenin chalcone (Sigma–Aldrich, St. Louis, MO, USA).

Total flavonoid and anthocyanin content

For total flavonoid contents, the samples were measured prior to HPLC injection at 361 nm by a NanoDrop photospectrometer (Thermo Scientific, Wilmington, DE, USA). We used rutin as the standard.

We measured anthocyanin content with minor modifications (Solfanelli et al. 2006). First, we ground tomato peels in volume HCl 0.5% (v/v) in methanol. An equal volume of chloroform was added to the extract to remove cholorphylls and the mixture was centrifuged for 1 min at 14,000g. Anthocyanins containing phase were recovered and absorption was determined spectrophotometrically at 544 nm with the NanoDrop (Lim et al. 2014). Delphinidin 3-Rutinoside was used as a standard (APin chemicals LTD, Abingdon, UK).

Antioxidant activity

We measured the antioxidant capacity using the modified 2, 20 -azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) or ABTS method [25, 29, 30]. Antioxidants were extracted with a 5 ml extraction solution [methonal/ethanol (70/29.5/0.5, v/v/v)] from 1 g tomato peel and flesh samples. We incubated the antioxidant-containing extract in darkness overnight at -20˚C. Subsequently, we centrifuged the solution at 1000 rpm for 2 min. An ABTS [(2.5 mM) (Roche Diagnostics, Indianapolis, IN, USA)] stock solution was prepared and approximately 0.4 g of MnO_2 (Acros Organics, Belgium) was added to the stock solution to generate ABTS radical cation (ABTS*). Excessive MnO_2 was removed using a 0.2 mM disk filter (Millipore Corp., Bedford, MA, USA). The ABTS* solution was then incubated in a 30˚C water bath and was diluted to an absorbance of 0.7 at 730 nm using a 5 mM phosphate buffer saline solution [pH 7.4 and ionic strength (150 mM NaCl)]. We then added 100 μL of the extract added to 1 mL of the ABTS* solution and vortexed for 10 s. Mixture absorbance was measured at 730 nm in a spectrophotometer (U-1100, Hitachi Ltd. Japan) after a 1-min reaction period. Trolox [(6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxyl acid) (Acros Organics, Belgium)] standard curve was prepared using a 0.5 mM stock solution.

Lycopene analysis

Lycopene analysis was modified from (Levin et al. 2003; Yodjun et al. 2011). Lycopene was extracted from the peel and flesh tissue of fresh red-ripe fruits. The peel was macerated to a powder in liquid nitrogen with a mortar and pestle. The flesh tissue was minced to a puree in a mortar and pestle. The extraction buffer consisted of n-hexane:isopropanol:acetone (2:1:1) and contained 0.05% BHT. The 1 g sample was stirred with 5 ml of an extraction buffer for 30 min in the dark. The extraction buffer consisted of a hexane:acetone:ethanol ratio of 2:1:1 (v/v/v) (Yodjun et al. 2011). Spectrophotometric absorbance at 472 nm was used to calculate lycopene concentration. The lycopene standard was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Stacking genes by cross-pollination

The anthers were removed from unopened flowers one day before anthesis. Before noon the next day, the pollen was collected with forceps from both CHI and DR plants. The emasculated flowers were pollinated with forceps. After pollination, the forceps were rinsed in a 70 % alcohol solution and wiped with a tissue to kill pollen for next use.

Harvesting and growing condition

The temperature was set to 28 ºC in the day and 24 ºC at night. The plantlets were potted in 4 gallon plastic pots with soil, Metro-Mix 700 medium (Sun Gro Horticulture, Agawam, MA). All the plants were pruned to have 3-4 branches. The 2 ~ 3 fruits were pooled to measure weight. The fruits were harvest in c.a. two month.

Statistical analysis

The statistical analysis for the categorical data was modified as described previously (Lim and Earle 2008; Lim and Earle 2009). The ANOVA (Analysis of variance) model was used. The analyses were performed using the GLM procedure (SASInstitute 2004). The interaction between the genes and the line was considered for the SAS model. The mean separation was performed by Tukey test. For repetition, each line had 4-6 plants. The two to three pooled tomatoes were collected from each plant for every lines.

Results

Generation of CHI- and DR-expressing tomato plants

More than 10 independent transgenic lines were generated from *CHI and Del/Ros* constructs, separately, and 2 - 3 morphologically normal and healthy lines were selected and subjected to further analysis. Prior to crossing to combine the genes, the segregation analysis was performed at T1 generation stage. The T1 lines showing 3:1 segregation ratio was chosen for further crossing to ensure the single transgene insertion. The segregation analysis was carried out on 100 mg⁄L kanamycin selection medium and confirmed by reverse transcriptase (RT)-PCR analysis (Fig. 2). The F1 CHI x DR is defined as the generation crossed by both T1 transgenic plants. All the stacked and unstacked genes were stably transmitted to the next generations.

The shape and weight of all the transgenic and crossed tomatoes were indistinguishable from those of the wild type plants (Fig. 3 a, b, c, Fig. 4 and Table 1). All of the T0, T1 and T2 and F1 and F2 transgenic and crossed lines developed as many seeds as the wild type plants (Fig. 3b). The outer peel color of the transgenic DR and CHI/DR tomatoes were purple and indigo, respectively (Fig. 3a and Fig 4 c and d). Also, the flesh color of the CHI/DR tomato was conspicuously darker than that of the DR tomato (Fig. 3c). The colors of the anthocyanin extracted from the peel and the flesh showed similar tendency with the peel and flesh color (Fig. 3d).

Total anthocyanin content in CHI/DR-expressing tomatoes

There was approximately maximum 100 times (0.8 ug/mg) and 400 times (3.25 ug/mg) more total anthocyanin content in the peel of the DR (T2) and CHI/DR lines (F1 and F2, Fig 5 a and b), respectively, as compared to wild type plants (0.008 ug/mg). There was approximately maximum 80 (c.a. 0.01 ug/ml) times and 260 times (c.a. 0.31 ug/mg) more total anthocyanin content in the flesh of the DR and CHI/DR lines, respectively than wild type (c.a 0.001ug/mg). The anthocyanin content was almost untraceable in wild type plants. There were no significant

differences between different transgenic lines for each gene. All the CHI/DR lines exhibited significantly higher anthocyanin content than all the CHI or DR alone transgenic tomatoes in both the peel and the flesh. Further, there were no significant differences between F1 and F2 generation for both the peel and the flesh. The total anthocyanin content of the wild type and CHI lines were barely traceable in both the peel and the flesh. Addition of the *CHI* gene to DR lines approximately increased total anthocyanin content in both peel and flesh 4 fold.

Total flavonol content in *CHI/DR*-expressing tomatoes

The total flavonol content exhibited considerable dynamic variation in both the peel and the flesh (Fig 5 c and d). For the peel, all the CHI/DR lines showed significantly higher flavonol content than the wild type and DR lines. The CHI-8 line has highest flavonol content than any other transgenic plants. The CHI-6 line has no significant flavonol differences with CHI/DR and it has higher flavonol content than DR and wild type lines. This implied that the substantial increase in total anthocyanin content might not occur at the expense of total flavonols.

Regarding the flesh, all the CHI/DR lines exhibited significant differences from all of the CHI and DR lines as well as the wild type plants. In flesh, the CHI/DR lines exhibited 150–200 times more total flavonol content (0.45 - 0.5 ug/mg) than the wild type plants (0.003 ug/mg), however the CHI and DR only lines approximately 20–27 times (0.06 – 0.08 ug/mg) and 50–60 times (average 0.15-0.18 ug/mg) than wild type plants., respectively. Addition of CHI to DR lines approximately increased total flavonol content 1.7 fold from 3.1 ug/mg in DR to 5.3 ug/mg in CHI/DR in peel and 2.8 fold from 0.18 ug/mg in DR to 0.5 ug/mg in CHI in flesh.

Flavonol composition of in *CHI*-, *DR*-, and *CHI/DR*-expressing tomatoes

Quercetin-3-B-D glucoside (QBD) and Rutin are both glycosylated forms of quercetin, and Kaempferol-3-Rutinoside is the glycosylated form of Kaempferol. In tomato peel, the QBD content from high to low was: CHI > DR > DR/CHI > wild type tomato, the rutin content from high to low was: CHI > CHI/DR > DR > wild type tomato, and the quercetin content from high to low was: CHI/DR > DR > CHI > wild type tomato (Fig. 6). Although these three chemicals contain the same aglycon, the content of them was not the same from plant transformed with

different genes. The quercetin content may have been left over from the glycosylation to QBD or rutin. The Kaempferol-3-rutisnoside (KR) content was highest in the CHI/DR lines, but the CHI and DR lines exhibited no differences between each other. CHI converts naringenin chalcone to naringenin. The ratio of naringenin chalcone vs. naringenin might indicate the efficiency of CHI as an indirect indicator of *CHI* expression. The content of these compounds in the DR and CHI/DR tomatoes exhibited variation among the lines. There was less naringenin chalcone than naringenin in the CHI tomato. Finally, the naringenin chalcone content of the CHI/DR lines fell between that of the CHI and DR lines caused by overexpression of the *CHI* gene in the CHI/DR lines during the pumping of the naringenin chalcone flux into naringenin. The naringenin content of the DR and CHI/DR lines was higher than in the wild type and CHI lines (Fig. 6).

Regarding the flesh, the QBD content of the CHI lines was significantly higher than that in the DR, CHI/DR, and wild type lines, but the CHI/DR line exhibited the highest rutin and KR content among all the transgenic lines (Fig. 7). Although quercetin, naringenin, and narigenin chalcone were detected in the peel, they were not detected in the flesh.

Lycopene content and antioxidant activity in *CHI/DR*-expressing tomatoes

In order to investigate whether *CHI*, *DR*, and *CHI/DR* expressing tomatoes affect lycopene production pathway, we measured total lycopene content. No significant differences of lycopene content were detected between any of the transgenic lines and the wild type fruit, and the color of the lycopene extracts in the wild type and transgenic plants was indistinguishable in both the peel and the flesh (Fig. 8a and b).

In the peel, a Trolox equivalent anti-oxidant capacity (TEAC) assay showed that all the CHI and CHI/DR lines exhibited 18–24 times more TEAC while DR increased by only 10 times as compared to wild type (Fig. 8c and d Thus, peels in CHI/DR lines exhibited more than twice antioxidant activity than DR only plants. In the flesh, the antioxidant capacity of the CHI/DR lines was significantly higher than that in the single-gene transgenic plants, CHI and DR. TEAC was 45% higher in flesh tissue of DR/CHI plants than in the same tissues of DR only expressing plants (DR-11). Other lines had 27-42% increase by crossing with CHI lines.

Fruit yield measurements

There was no statistical difference between lines in every gene and between genes for weight and numbers of fruit. The average of fruit weight and number of fruit which were harvested in given time for across all lines were 49.1 (±5.03) and 36.9 (±5.97).

Discussion

In this study, we generated dark purple tomatoes with approximately 4-fold and 2-fold increases in the levels of anthocyanins in tomato peel and flesh, respectively, compared to the *DR*-expressing tomatoes after co-expression of *CHI* in the *DR*-expressing tomato plants (Fig 5a). This result suggests that the conversion of naringenin chalcone to naringenin, catalyzed by CHI, is still a bottleneck in the anthocyanin biosynthesis pathway, though DR in tomatoes induce the expression of endogenous *CHI*. In addition to increased anthocyanins, we also observed a significant increase in total flavonol content after the addition of *CHI* to *DR*-expressing lines (Fig 5c) in tomato peel. With respect to flavonol content in tomato peel, it seems the DR may have less impact on total flavonol accumulation than CHI alone lines, which was confirmed by comparing flavonol content from CHI and CHI/DR lines (Fig 5c). The CHI alone lines increased the flavonol and anthocyanin content in peel by average 8 and 1.4 fold,respectively, however the DR alone lines increased them by only 4 and 77 fold (Fig 5), respectively.It is probably due to the tendency of DR to shift the pathways towards anthocyanin production and away from other flavonol production.

Compared to peel tissue, it is hard to achieve substantial increase of anthocyanin content in flesh. However, several groups reported flavonol increase in flesh by overexpressing some genes. Colliver (2002) (Colliver et al. 2002) reported that concomitant expression of *CHI, CHS, F3H* and *FLS* in tomato flesh increases flavonol content. Overexpression of *CHI, CHS, and F3H* led to an increase of dihydrokaempferol, which is one major branch point between anthocyanins and flavonols. Overexpression of *FLS* ensured a major shift towards the flavonol pathway and an increase in kaempferol, a flavonol. Among the 4 genes, *CHS* plays key role for flavonol production in flesh (Colliver et al. 2002). There was no report that *Del/Ros* could upregulate *CHS* or *FLS* (Butelli et al. 2008). Interestingly, the flesh of our DR overexpressing tomatoes was visibly purple in this experiment without upregulation of *CHS* and *FLS* (Butelli et al. 2008), even

though it can act as a reportedly bottleneck (Butelli et al. 2008). This suggests that while *CHS* is an important bottleneck early in the pathway, it may not be as important as upregulation of genes dedicated to anthocyanin production such as *DFR, ANS, 3-GT,* and *5-GT* for increased anthocyanin content. That indicates there may already be sufficient substrate available for anthocyanin production after upregulation of *PAL*, *CHI*, and *F3H*. Our *CHI* gene produced a moderate increase in both flavonol and anthocyanin content in the flesh after stacking DR genes. Reportedly, the *DR* gene upregulates not only *CHI*, but also *PAL*, and *F3H* (Butelli et al. 2008). All these genes play a role in conversion of phenylalanine into flavonol precursors known as dihydroflavonols. Dihydroflavols occur at the branching point between anthocyanin and flavonols.

Butelli et al (2008) reported that *CHI* activity approximately doubled in the turn/ripe state. Compared with *PAL* activity upregulated by *DR*, this increase in *CHI* activity is insufficient to fully reap the benefits of an upregulated PAL. Luo (2008) reported a similar situation that the content of rutin and kaempferol rutinoside showed no obvious differences between them even though the *AtMYB12* gene upregulated *F3'H* to move the flux from dihydrokaempferol to dihydroquercetin (Figure 1). In our experiment, the flavonol rutin is the most abundant flavonol in all wild type, *CHI*, *DR*, and *CHI/DR* lines. *DR* upregulates *F3'5'H*, which converts dihydrokaempferol to dihydromyricetin (Butelli et al. 2008). This causes a competition between rutin production and anthocyanin production. Dihydrokaempferol is a common substrate of *FLS, F3'H,* and *F3'5'H* (Fig 1). Despite the competition for the common substrate, the *DR* and *CHI/DR* lines exhibit the highest rutin content compared with other flavonols, which suggests that *CHI* overexpression provides more than enough substrate for one enzyme to fully utilize. One of the main anthocyanins in the *DR* lines is a delphinidin type of which the precursor is dihydromyricetin (Butelli et al. 2008). Bovy et al. (2002) reported that in *Solanaceous* species, *DFR* prefers dihydromyricetin and will not utilize dihydrokaempferol as a substrate. Dihydromyricetin is the precursor to the delphinsoluidin-type anthocyanins located far upstream along the flavonoid pathway and utilizes *DFR* to move in that direction. This indicates that, while F3'5'H activity drastically increases anthocyanin production by providing ample substrate, its activity is not so high that all dihydrokampferol is converted to dihydromyricetin. Thus, there is still enough substrate available to be converted to dihydroquercetin and eventually rutin, which suggests that *CHI* overexpression provides ample substrate for significant increases

in both flavonoid and anthocyanin production. We used an ethanol/methanol extract to measure antioxidant activity. The water extraction property of flavonoids poorly reflects the effect of rutin on antioxidant activity because rutin is insoluble in water. The wild type, and transgenic tomatos are a good source of lycopene and exhibit strong antioxidant activity (Ozkan et al. 2012). Lycopene, including β-carotenoid, is hydrophobic, unlike most flavonoids. The lycopene content in all wild and transgenic lines exhibited no significant differences, implying that intrinsic antioxidant activity in tomatoes remains intact even if when achieving a substantial increase in soluble flavonols and anthocyanin.

Several strategies have previously been reported for increasing flavonoid content. Until now, all attempts to increase flavonoid levels have focused on one flavonol or anthocyanin end products. *Lc/C1* ectopic expression does not upregulate the *CHI* and *PAL* genes, but it results in a moderate increase in both flavonol and anthocyanin (Bovy et al. 2002). *DR* upregulation only led to increased anthocyanin content (Butelli et al. 2008). *AtMBY12* upregulates the genes that are necessary for flavonol production, except for *DFR*, which is the first step in anthocyanin production and results in increased flavonol production. By adding CHI to DR expressing tomato plants we achieved an increase in anthocyanin and total flavonol content by alleviating a major bottleneck and providing ample substrate for the increased production of both. Combining CHI and DR, all three genes to maximize the capacity of CHI results in 4 fold increase in both peel and flesh in DR only tomato without the expense of flavonol content which is competing for common precursor.

Author's contribution WL conceived the study, planned experiments, performed experiments and collected data. WL and JL wrote the manuscript. WL and JL analyzed the data, and edited the manuscript.

Acknowledgments The research was supported by HI Green System in Korea

Compliance with ethical standards The authors comply with all ethical standards

Conflict of interest The authors declare no competing financial interests.

Figure legends

Fig.1 Schematic representation of the flavonoids biosynthetic pathway. PAL: phenylalanine ammonia lyase; 4CL: 4-coumarate:coenzyme A ligase; C4H: cinnamate 4-hydroxylase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone-3-hydroxylase; F3'H: flavonoid-3'-hydroxylase; F3'5'H: flavonoid-3'5'-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; 3-GT: flavonoid 3-O glucosyltransferase; 5-GT: flavonoid 3-O glucosyltransferase; AAC: anthocyanin acyltransferase; RT: flavonoid 3-O-glucoside-rhamnosyltransferase.

Fig.2 Schematic expression of Reverse trascriptase PCR (RT-PCR) for each CHI/DR line, in peel and flesh: (a) CHI primer (b) Del primer (c) Ros primer (d) Housekeeping gene primer, PePP2ACS, which is used for equal loading.

Fig.3 Color expression in T2 and F2-generation tomatoes (cv. Rubion), expressing , CHI and CHI/DR or both: (a) whole fruit (b) dissected fruit with seeds (c) dissected fruit without seeds (d) extracted anthocyanin

Fig. 4 Fruits in plant and whole plant (a) Wild (b) CHI (c) DR (d) CHI x DR (e) and (f) wild, DR, CHI x DR and CHI from left, (a) – (d) show various ripening states

Fig. 5 Total Anthocyanin content in (a) peel and (b) flesh and total flavonol content in (c) peel and (d) flesh (CHI lines have T2 generations.). Values with the same letter are not significantly different at 0.05 using the Tukey test. Tomatoes were harvested 20 d after breaker stage. The data represent the mean values (±SD) derived from 5-7 plants per each line (4 to 6 pooled tomatoes per plant).

Fig. 6 Flavonols from skin: (a) quercetin-3-B-D glucoside (b) rutin (ckaempferol-3-rutinoside (d) quercetin (e) naringenin (f) naringenin chalcone (F2 CHI/DR population). Values with the same letter are not significantly different at 0.05 using the Tukey test. Tomatoes were harvested 20 d after breaker stage. The data represent the mean values (±SD) derived from 5-7 plants per each line (4 to 6 pooled tomatoes per plant).

Fig. 7 Flavonol from flesh: (a) Quercetin-3-B-D glucoside (b) Rutin (c) Kaempferol-3-Rutinoside. Values with the same letter are not significantly different at 0.05 using the Tukey test. Tomatoes were harvested 20 d after breaker stage. The data represent the mean values (±SD) derived from 4 plants per each line (4 to 6 pooled tomatoes per plant).

Fig. 8 . Lycopene content in (a) peel and (b) flesh and antioxidant activity in (c) peel and (d) flesh. Tomatoes were harvested 20 d after breaker stage. The data represent the mean values (±SD) derived from 5-7 plants per each line (4 to 6 pooled tomatoes per plant).

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Key message

Stacking of a structural gene, chalcone isomerase and two regulatory genes, Del and Ros1 shows synergistic effects to improve nutrition quality of tomato by increasing both anthocyanin and flavonol contents.

Figure 1

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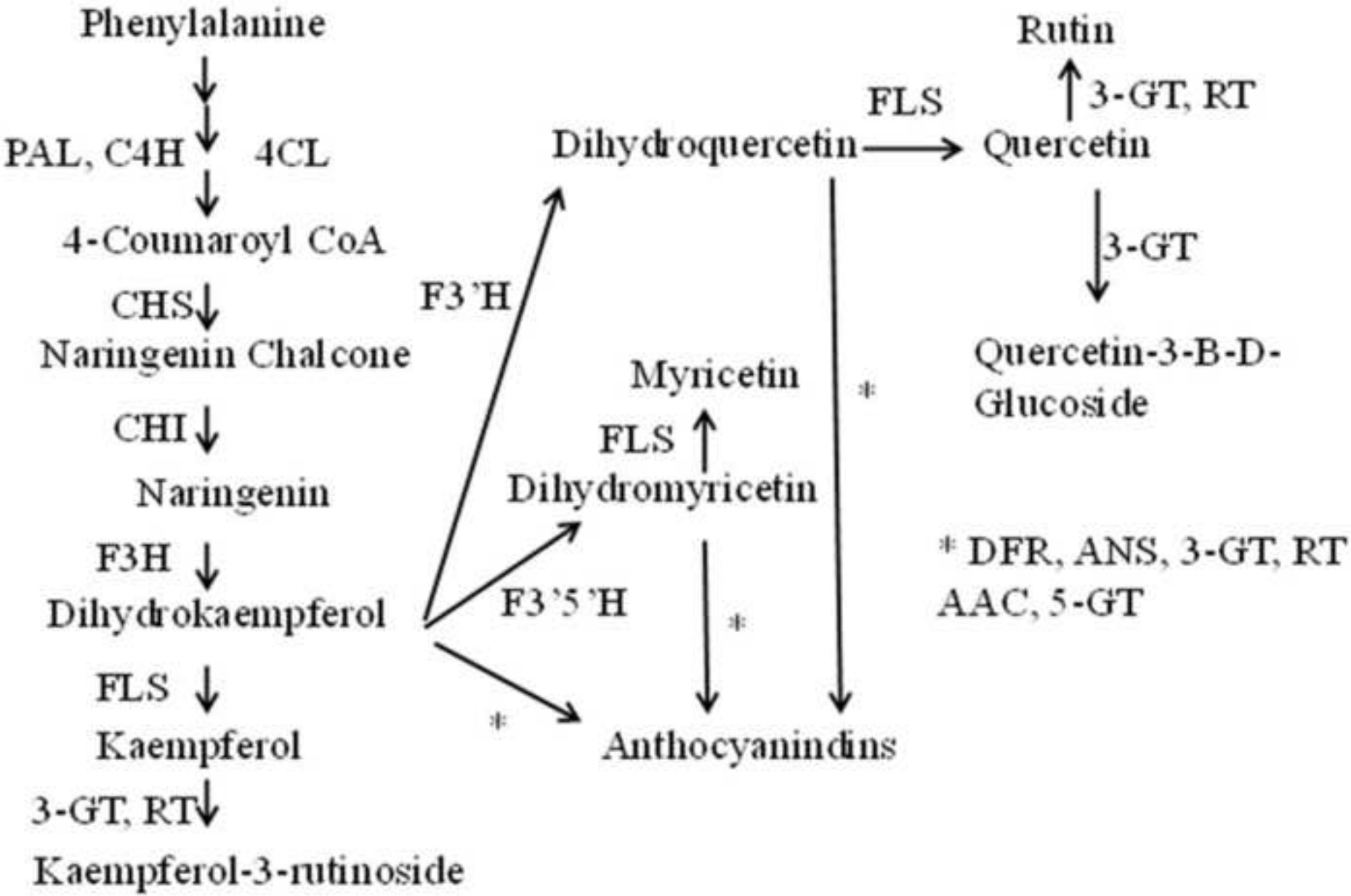

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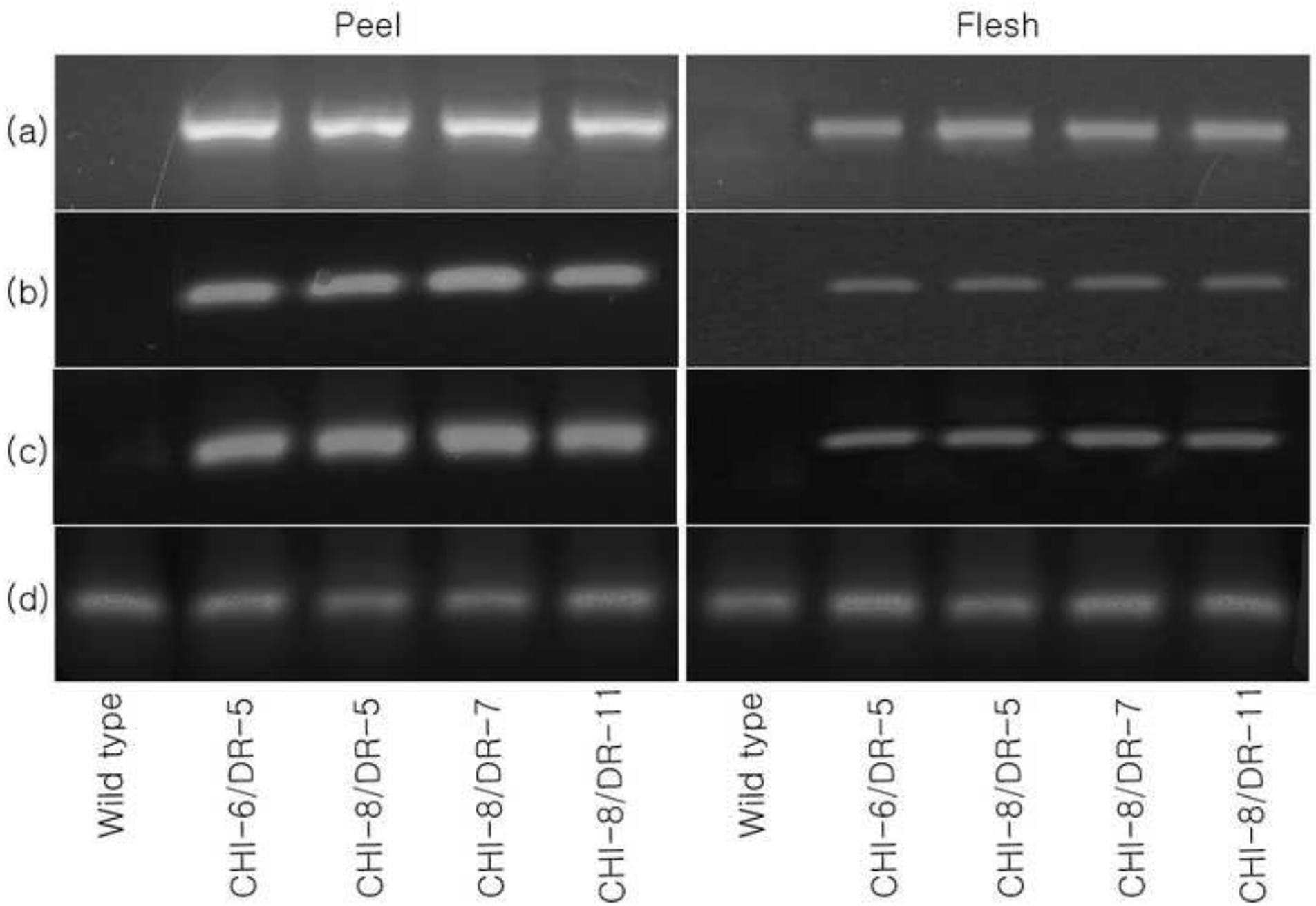

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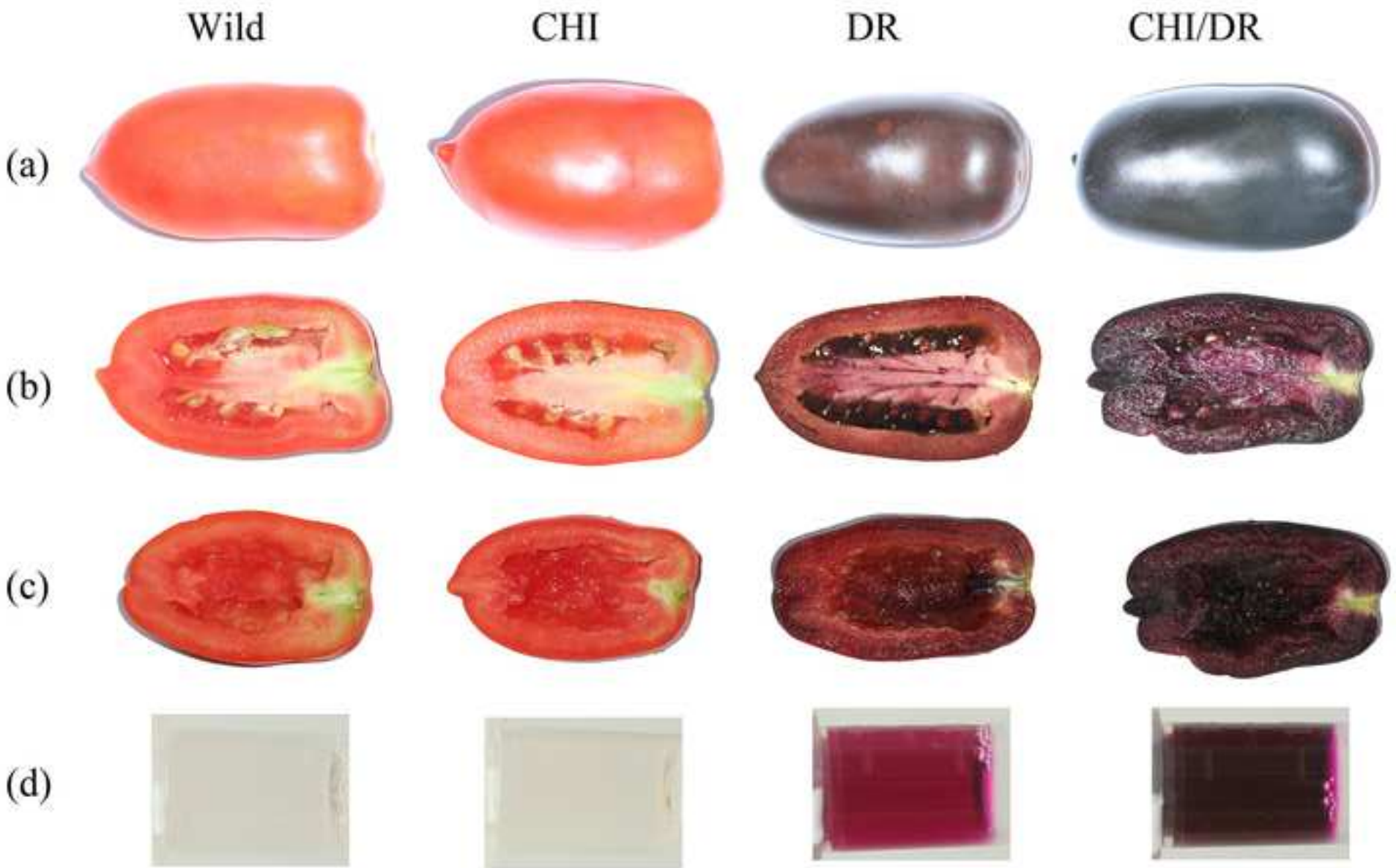

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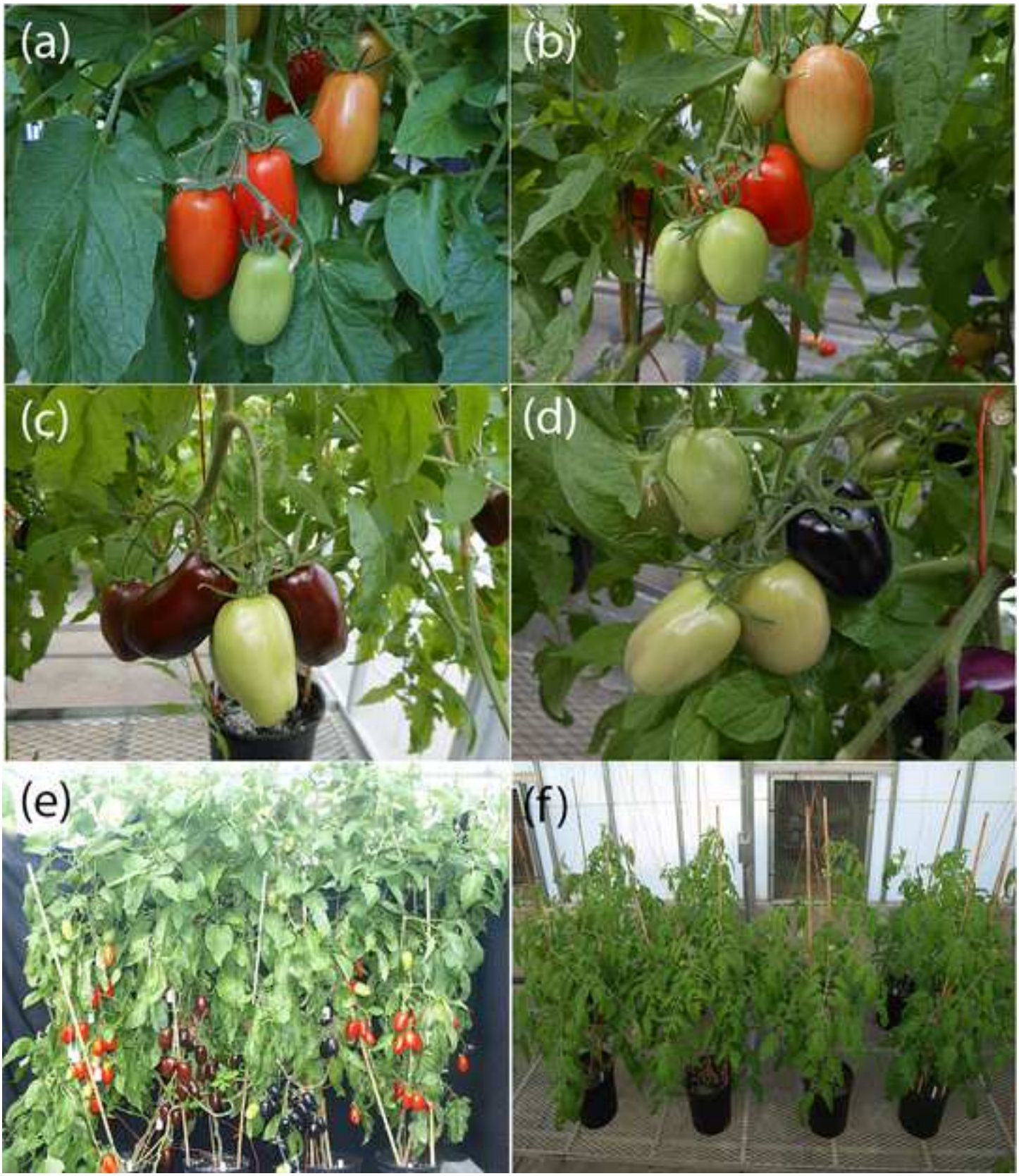

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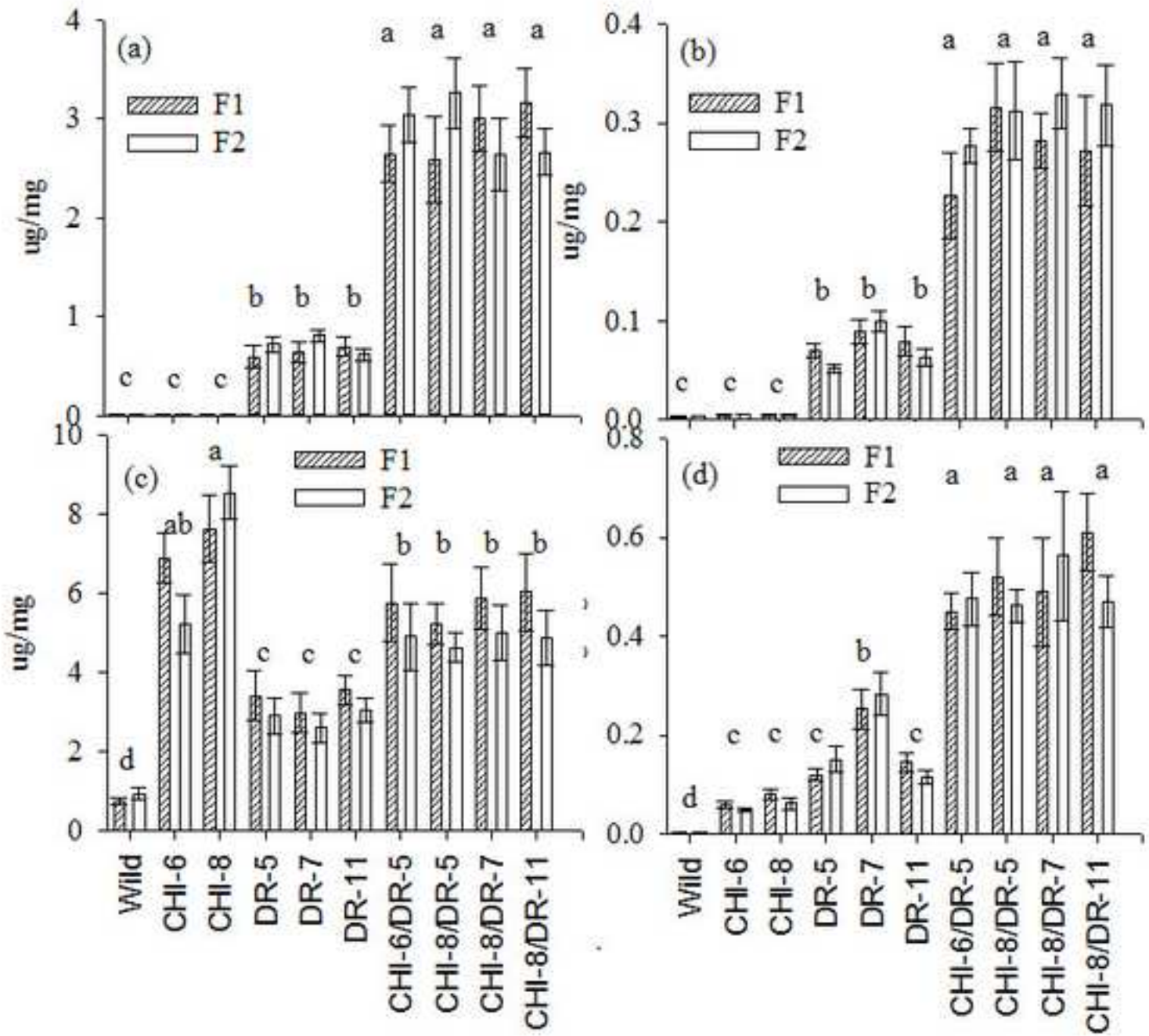

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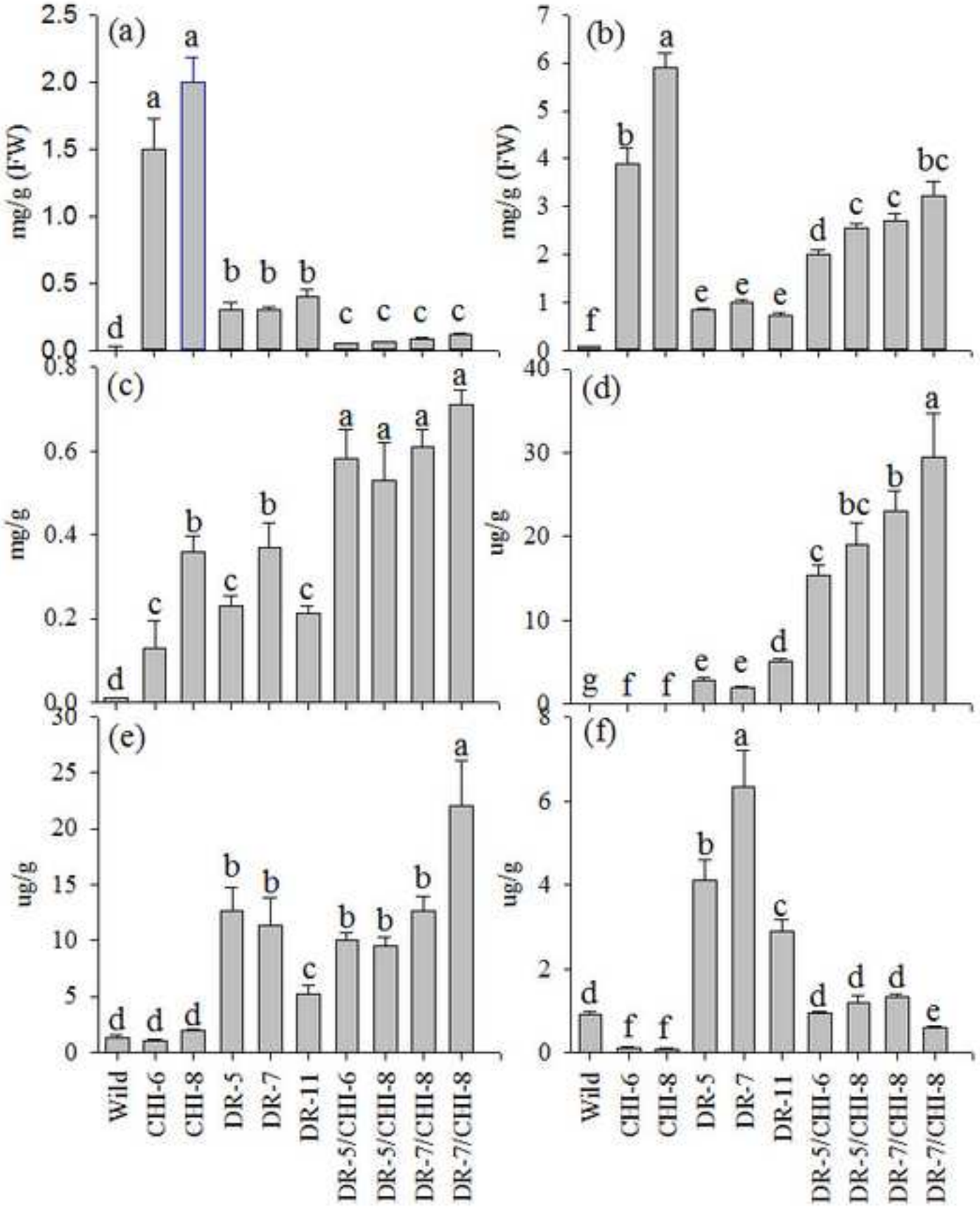

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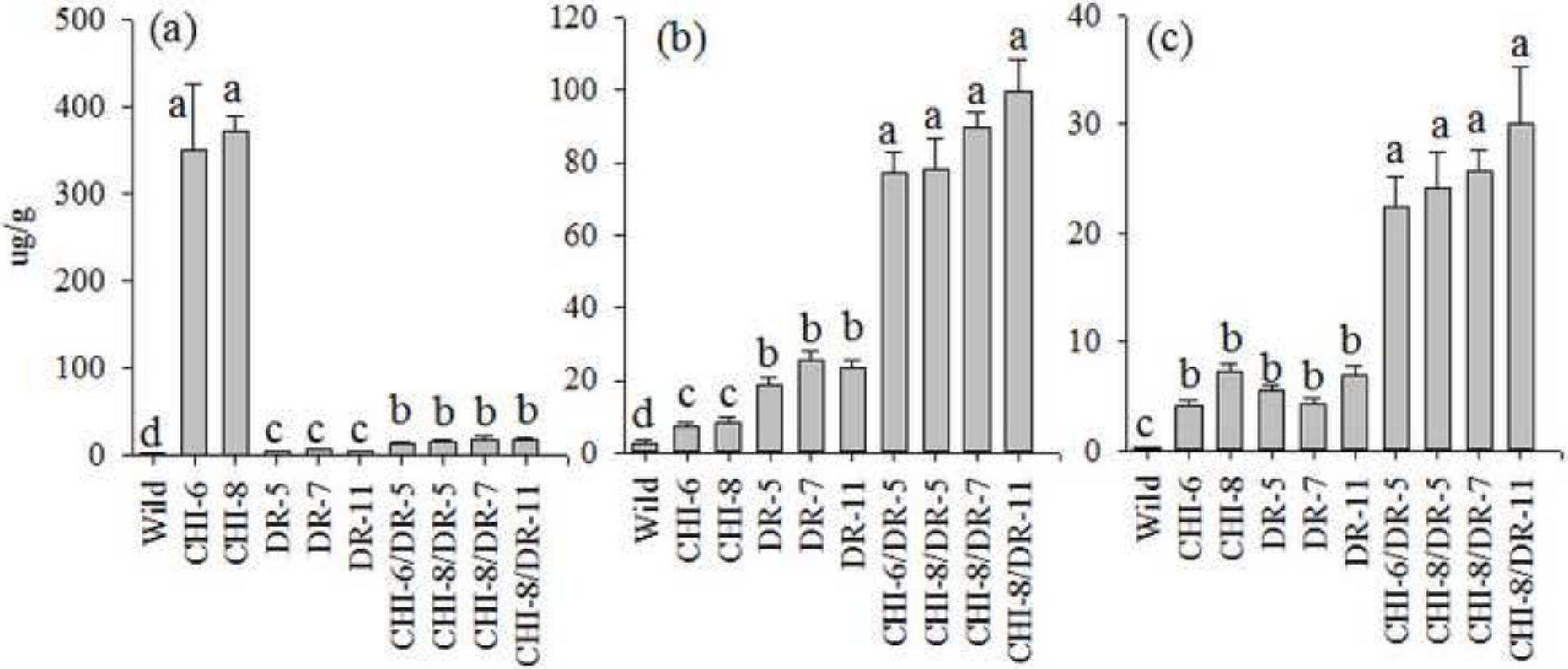

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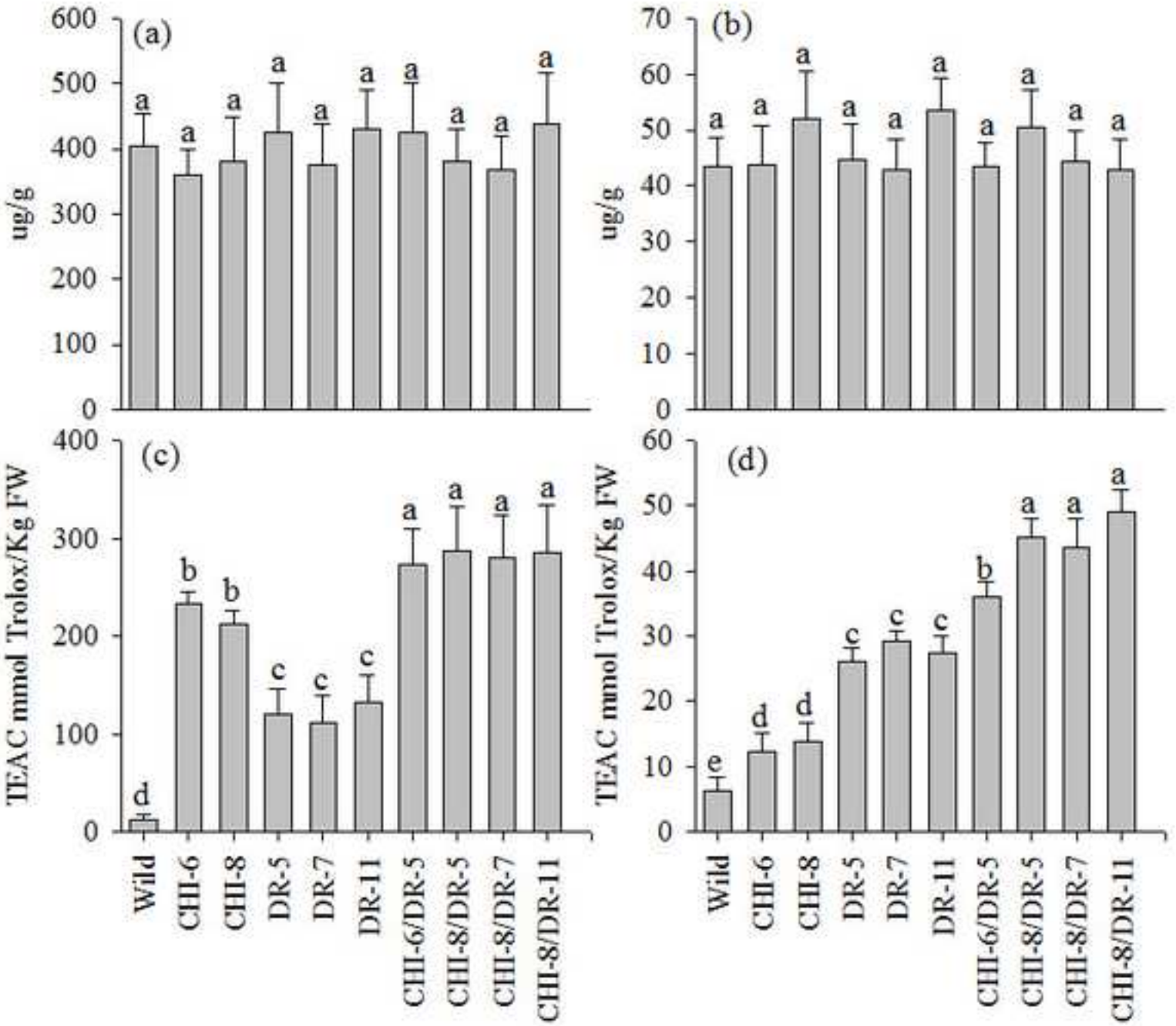

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Table 1. The weight of individual fruit and number of harvest fruit per plant. Each line has 5 – 7 replication. The individual plant was pruned to have 3 – 4 branches[a].

Gene	Line	Weight		Number per plant	
		Mean	Standard deviation	Mean	Standard error
Wild		50.8	± 4.2	37.7	± 6.7
CHI	4	52.0	± 4.4	39.0	± 2.6
	8	46.6	± 4.8	31.7	± 8.4
DR	5	47.0	± 4.9	40.3	± 7.4
	7	43.6	± 4.5	30.5	± 4.9
	11	46.4	± 4.2	42.0	± 6.9
CHI/DR	C-6/DR-5	51.4	± 6.1	33.4	± 5.3
	C-8/DR-5	46.0	± 6.5	34.7	± 4.2
	C-8/DR-7	52.8	± 5.5	35.2	± 6.2
	C-8/DR-11	54.4	± 5.2	44.6	± 7.1

[a]Mature fruits were harvested from T3 for CHI and homozygous F2 populations of three independent transgenic lines. Tomatoes were harvested 20 d after breaker stage. The data represent the mean values (±SD). There was no statistical difference in ANOVA test.

Respond Letter

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